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(54) Title: ANTHRAX CONJUGATE VACCINE AND ANTIBODIES AGAINST BACILLI AND ANTHRAX TOXINS

(57) Abstract: The present invention is directed to immunogenic conjugates comprised of poly-γ-D-glutamic acid (PGA) which has been covalently bound to protective antigen (PA). The invention includes methods for making conjugates, vaccines in which they are present and methods for immunizing individuals in which they are used. Conjugation produces a synergistic effect dramatically increasing the response of animals to both the PGA and PA components of the conjugate. Antibodies to PGA and PA confer protection against bacilli and anthrax toxins.

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Anthrax Conjugate Vaccine and Antibodies Against Bacilli and Anthrax Toxins

Cross Reference to Related Application

The present application claims the benefit of U.S. provisional application no. 60/461,406, filed on April 10, 2003.

Field of the Invention

The present invention is directed to a conjugate vaccine that induces an immune response against both pathogenic *Bacillus* species and anthrax toxin. The invention includes methods by which the conjugate is made, vaccines containing the conjugate and methods of inducing an immune response by administering the vaccines to an individual.

Background of the Invention

Anthrax is a relatively rare bacterial infection that may be caused by inhalation, skin contact or gastrointestinal absorption. When exposed to the air, the bacteria that causes anthrax forms spores which may persist for decades and cause disease if inhaled. Since infection is difficult to diagnose in its early stages and is usually fatal if not promptly treated with antibiotics, anthrax has been favored as a biological weapon since the early 1900's.

Although anthrax infections may be treated with antibiotics, vaccines are generally considered to be a preferable means of protecting populations. At present, three types of vaccine are commercially available: a Georgian/Russian vaccine, a U.K. vaccine and a U.S. vaccine. The Georgian/Russian vaccine is associated with a large number of side effects and contraindications. The U.K. and U.S. vaccines are somewhat safer but their effectiveness varies considerably from one preparation to the next. In addition, multiple injections must be given over a period of 18 months, followed by a yearly booster, in order to attain and preserve immunity. Because of terrorist activities in the United States and elsewhere, there is considerable interest in the development of an improved version of these vaccines.

The ability of anthrax to cause a fatal infection depends upon its bacterial capsule and the presence of a toxin complex. The capsule is made of poly-γ-D-glutamic acid and protects the bacteria against leukocyte phagocytosis and lysis, which allows the unimpeded

multiplication of bacilli in the host (Dixon, *et al.*, *N. Eng. J. Med. 341*:815 (1999)). Bacterial isolates lacking capsules are virtually avirulent (Welkos, *Microb. Pathog. 10*:183 (1991)). Although the capsule is of critical importance in bacterial infection, it is only weakly immunogenic and has therefore generally not been considered as a good target antigen for vaccines.

The toxin produced by anthrax bacteria is also necessary for a fatal infection and is composed of three entities: a "protective antigen," a "lethal factor," and an "edema factor" (Dixon, *et al.*, *N. Eng. J. Med. 341*:815 (1999); Lacy, *et al.*, *Curr. Top. Microbiol. Immunol. 271*:61 (2002)). The protective antigen is not, in itself, toxic but it binds to membrane receptors and allows the entry of the other two agents into cells. Because of its ability to safely elicit a protective immune response against anthrax toxins, the protective antigen has been a favorite target in vaccines. However, these vaccines do nothing to stop the replication of bacteria.

Overall, an ideal anthrax vaccine would be safe, consistently effective and capable of targeting both bacteria and bacterial toxins.

Summary of the Invention

The present invention is based upon the development of a dually active anthrax vaccine (DAAV) that confers simultaneous protection against both bacilli and toxins. The vaccine utilizes a conjugate protein in which capsular poly-γ-D-glutamic acid (PGA) is covalently bound to protective antigen (PA). The PGA-PA conjugation converts the weakly immunogenic PGA into a potent immunogen and synergistically enhances the humoral response to PA. DAAV provides both prophylactic (antibacterial) and therapeutic (antitoxin) components in a single vaccine. Sera from DAAV-immunized mice were able to specifically bind to PGA-encapsulated bacilli and promote the killing of the bacilli. Sera from DAAV-immunized mice were also able to neutralized anthrax toxin activity. Mice immunized with this vaccine were protected against challenge with anthrax lethal toxin.

In its first aspect, the invention is directed to a method of making a conjugate that is useful in the induction of an immune response against infection by anthrax. The method involves covalently coupling PGA with PA to produce a conjugate that induces a stronger

immunological response than either component used alone. The PGA can be made either synthetically or produced from bacteria using procedures such as those described in the Examples section below. It should have a molecular size of between of PGA from 0.2 kDa and 500 kDa, preferably between 0.5 kDa and 350 kDa and still more preferably between 20 kDa and 200 kDa. PA can be obtained using methods that have been described in the art. The ratio of PGA to PA should be between 1:0.05 (w/w) and 1:20 (w/w), preferably between 1:0.1 and 1:10 (w/w) and more preferably between 1:0.5 and 1:3 (w/w). In one particularly preferred embodiment, the PGA used in making conjugates has a molecular size of between 80 and 120 kDa and the PGA to PA ratio is 1:2 (w/w). The method is compatible with any procedure for conjugating the PGA and PA components together. For example, coupling can be accomplished using reagents that facilitate the coupling of carboxylate and amino groups, such as carbodiimide-containing agents. One agent that has been found to give good results is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Other example coupling reagents include 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide, dicyclohexyl carbodiimide, and diisopropyl carbodiimide, N,N'-carbonyldiimidazole, and Woodward's reagent K (N-ethyl-3-phenylisozazolium-3'-sulfonate). The coupling of PGA and PA may also accomplished through linker molecules, *i.e.*, by coupling both PGA and PA to a third molecule. It is also possible to couple imunogenic proteins such as those described below to the PGA along with PA.

In another aspect, the invention is directed to the conjugates themselves. The main defining characteristic of these conjugates, besides the presence of PGA and PA in the sizes and ratios described above, is their ability to induce a strong immunological response against both the anthrax bacteria and the anthrax toxin. Using a mouse model of infection, conjugates were found to confer protective immunity against challenge with intact anthrax toxin that would otherwise have been lethal.

The invention also includes vaccines comprising the conjugate described above in a pharmaceutically acceptable carrier. The conjugate should be present at a concentration such that it is effective upon administration of one or more unit doses of the vaccine to induce an immune response. As defined herein, an immune response means inducing the synthesis of antibodies against both anthrax bacteria and anthrax toxin and/or inducing a cell-mediated

immunological response. The vaccine may optionally also include other antigens against either anthrax bacteria or toxin, an antigen against a different pathological agent or adjuvant.

The present invention encompasses methods of inducing an immune response in an individual (human or animal, particularly sheep) by administering the vaccines described above. The dosage administered should be sufficient to induce a strong immunological response and should, in general, be in the range of between 10 micrograms and 10 mg. The vaccine may be administered once or in a multi-week regimen similar to vaccines presently on the market. Procedures for determining optimum concentrations and dosage schedules are well known in the art and can be applied to DAAV.

Apart from DAAV, it has been found that a much stronger immune response can be generated from PGA when it is conjugated to an immunogenic protein such as hepatitis B core antigen protein, tetanus toxoid, or diphtheria toxoid. An "immunogenic protein" as defined herein refers to a protein that enhances an immune response when joined to an antigen and administered to a human or animal. Thus, the PGA-protein conjugates may be present in vaccines such as those described above and used to immunize individuals. Antibody preparations (serum or purified antibodies) may be obtained from humans or animals given the conjugates and then used in treatment methods. For example, an effective dose of antibody preparation may be given for the purpose of preventing or treating infection by bacilli such as anthrax. An effective dose, as used in this context, refers to sufficient antibody preparation to have a positive clinical effect as determined using standard parameters well known in the art.

Brief Description of the Drawings

Figure 1: Specific antibody response to DAAV-1 immunization in mice.

Anti-PGA IgG (graph A) and IgM (graph B). Anti-PGA IgG concentrations after the 3rd dose were significantly higher than after the 2nd ($p=0.002$, 10-μg group; $p=0.007$, 20-μg group). Anti-PA IgG (graph C) and IgM (graph D). After three doses, DAAV-1 induced significantly more anti-PA IgG than PA alone ($p=0.02$, 10-μg group vs. PA; $p=0.01$, 20-μg group vs. PA). Note that all anti-PA IgM concentrations are very low and close to the detection limit. No significant difference exists between 10- and 20-μg groups (graphs A-D).

Control immunization agents included PBS, PGA, PA, and unconjugated PGA/PA mixture (mix). Boxes represent 25th to 75th percentiles of 8 data points and bars indicate minimum, median, and maximum values.

Figure 2: Specific antibody response to DAAV-2 immunization in mice:

Anti-PGA IgG (graph A), anti-PGA IgM (graph B), anti-PA IgG (graph C), and anti-PA IgM (graph D). Both anti-PGA and anti-PA IgG show booster responses. With respect to IgG, the 10- and 20-μg doses are superior to the 2-μg dose ($p<0.05$). The maximum attainable IgG concentrations are ~2-fold (anti-PGA) and ~10-fold (anti-PA) below those of DAAV-1. PBS served as a negative control. Boxes represent 25th to 75th percentiles of 8 data points and bars indicate minimum, median, and maximum values.

Figure 3: Anti-PGA IgG (left) and IgM (right) responses in mice immunized with a conjugate of PGA to hepatitis B core protein (con) and control PGA. Note that the conjugate induced significant levels of antibodies to PGA.

Figure 4: Antibacterial activities of anti-PGA antibodies.

Bactericidal activity of anti-PGA antibodies against *B. licheniformis* 9945a. Bacilli were incubated with complement and serial dilutions of sera from DAAV-immunized mice. Percentage of killing was calculated relative to growth of bacilli incubated with control preimmune serum and complement.

Figure 5: Protective activities of anti-PA antibodies.

Correlation between anti-PA IgG levels and protection of CHO cells against PA-mediated cytotoxicity. 0% and 100% levels correspond to cells incubated without serum or without LF_N-DTA, respectively (graph A). Preimmune sera or sera from mice immunized with PBS did not inhibit cytotoxicity. Protection of mice immunized with DAAVs or control agents against lethal toxin (LeTx) challenge (table B). Surviving mice were monitored for at least 2 weeks.

Figure 6: Amino acid sequence of Anthrax Protective Antigen.

Figure 6 shows the amino acid sequence of the protective antigen of the Anthrax toxin complex. The sequence is also shown in the sequence listing as SEQ ID NO:1.

Detailed Description of the Invention

The present invention is concerned with a conjugate between PGA and PA that produces a synergistic effect with respect to the immunological response generated against each of the conjugate components. The PGA used in the conjugates can be obtained synthetically or, alternatively, can be purified from bacterial cultures using well established procedures. One preferred method for obtaining PGA is described in the Examples section and utilizes *B. licheniformis*. In order to maintain the solubility of conjugates, ideally, the average molecular weight of the PGA should be less than 500 kDa. One way to reduce polymer size is through ultrasonic irradiation as described herein, but other methods should work equally well. PA may be obtained recombinantly (see Examples) or purified using methods known in the art. The full-length sequence of the protein has been previously described and is shown as Figure 6.

To induce antibodies to PGA, PGA may be coupled to other immunogenic protein carriers, such as hepatitis B core antigen protein (see Example), tetanus toxoid, and diphtheria toxoid. Such conjugates and the PGA-specific antibodies elicited by these conjugate may be used in active immunization or therapeutic settings to protect against infection caused by PGA-encapsulated *Bacillus* pathogens, including example *B. anthracis*, *B. licheniformis*, *B. cereus*, and *B. subtilis*. PGA-based conjugates and PGA-specific antibodies may be especially valuable in veterinary medicine.

To achieve protection against both bacilli and anthrax toxins, PGA-protein conjugates may be combined with PA to formulate a combination vaccine. In such combination vaccine, the PGA-protein conjugate is used to elicit antibodies to PGA, whereas PA is used to elicit antibodies to PA.

Methods of covalently coupling antigens and polymers have been described in scientific articles (see, *e.g.*, Jacob, *et al.*, *Eur. J. Immunol. 16*:1057-1062 (1986); Bessler, *Immunobiol. 170*:239-244 (1985); Posnett, *et al.*, *J. Biol. Chem. 263*:1719-1725 (1988); Ghose, *et al.*, *Molec. Immunol. 25*:223-230 (1988)) as well as in patents and published patent applications (EP 245 045; US 4,673,574; US 4,902,502; US 4,830,852; US 4,761,283; US 4,789,735; EP 206 852; US 4,619,828; and US 4,284,357). Any of these or similar

methods may be applied to the present invention. The most preferred method for making conjugates involves the use of a carbodiimide linker as discussed herein.

The conjugates described above may be formulated into vaccines optionally containing salts, buffers or other substances designed to improve the composition. Sterile solutions, *e.g.*, phosphate buffered saline, may be used as the carrier and preservatives may be added to improve the stability of preparations during storage. Adjuvants should not normally be needed but may be used if desired. These would normally be mixed with compositions prior to administration but separate presentation to a subject may also be used. Adjuvants can take the form of oil-based compositions, *e.g.*, Fruend's complete and incomplete preparations, mineral salts, *e.g.*, silica, kaolin, or carbon, polynucleotides or saponins. Examples of suitable materials for use in vaccines and methods for formulation are provided in Remington's Pharmaceutical Sciences (pp. 1324-1341, Mack Publishing Co., Easton, PA (1980)).

Vaccines or conjugates may be stored in a lyophilized form and reconstituted in a pharmaceutically acceptable carrier prior to administration. Alternatively, preparations can be stored in solution. The volume of a single dose, *i.e.*, a unit dose, of the vaccine will vary but, in general, it should be between about 0.1 ml and 2.0 ml and more typically between 0.2 ml and 1.0 ml.

Any method for administering the vaccines to a patient which does not result in the destruction of immunogenic conjugates is compatible with the present invention. Generally, administration will be by parenteral means such as intramuscular, subcutaneous or intravenous injection. The dosage and scheduling of administration of vaccines can be determined using methods that are routine in the art. In general, it is expected that vaccines prepared by the methods disclosed herein will contain from 10 μg/ml to 10 mg/ml per dose, and preferably between 50 and 500 μg/ml per dose. However, dosages higher or lower than these ranges are also compatible with the invention. The preparations may be administered by either single or multiple injection.

Examples

Systemic anthrax involves unimpeded bacilli replication and toxin secretion. The present example describes the development of a dually active anthrax vaccine (DAAV) that confers simultaneous protection against both bacilli and toxins. As described further below, DAAV was constructed by conjugating capsular poly-γ-D-glutamic acid (PGA) to protective antigen (PA). Conjugation converted the weakly immunogenic PGA to a potent immunogen and synergistically enhanced the humoral response to PA. Anti-PGA antibodies destroyed bacilli by opsonization and complement-mediated lysis. Anti-PA antibodies neutralized toxin activity and protected immunized mice against challenge with anthrax lethal toxin. Thus, DAAV provides both prophylactic (antibacterial) and therapeutic (antitoxic) components in a single vaccine.

A. Material and Methods

Strain, inoculation, and culture methods

B. licheniformis ATCC 9945a was obtained from the American Type Culture Collection (Atlanta, GA). Highly mucoid colonies were selected and grown aerobically in Erlenmeyer flasks with E broth (Birrer, *et al.*, *Int. J. Biol. Macromol. 16*:265-275 (1994)). The formulation of E medium in g/l was as follows: L-glutamic acid, 20.0; citric acid, 12.0; glycerol, 80.0; NH_4Cl, 7.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.04; $CaCl_2 \cdot 2H_2O$, 0.15; $MnSO_4 \cdot H_2O$, 0.104. Cultures were incubated at 37°C and shaken at 250 rpm for 96 h.

Purification of PGA

PGA was purified from the culture supernatant following the procedure described by Perez-Camero *et. al.* (Perez-Camero, *et al.*, *Biotechnol Bioeng 63*:110-115 (1999)) with slight modifications. The highly viscous bacterial culture was centrifuged at 4°C (6,500 g, 20 min) to remove bacteria. The supernatant was collected and precipitated with 3 vol of ethanol at 4°C overnight. PGA precipitate was collected by centrifugation and dialyzed against deionized water. PGA solution was acidified to pH 1.5 with 6 M HCl and immediately precipitated with 3 vol of 1-propanol at -20 °C. PGA was collected by centrifugation and washed twice with acetone and once with ethyl ether. The purified PGA was then dissolved in water, dialyzed extensively, and lyophilized. The purity and structure of PGA was verified by UV-Vis scanning from 190-300 nm and ^1H NMR spectroscopy.

Degradation of PGA by ultrasonic irradiation

The average molecular size of native PGA was about 500 kDa as determined on a Superose 12 column that had been calibrated with dextran standards. Because of the large size of PGA, direct conjugation to PA would result in an insoluble gel which is undesirable for vaccine formulation. Therefore, we degraded PGA by microwave irradiation using an ultrasonic processor (Tekmar, Cincinnati, OH) operating at 20 kHz (2). PGA solution (6.5 mg ml^{-1} in PBS) was placed in an ice bath to maintain the system at room temperature. The PGA solution was irradiated for 1.5 h and the average molecular size of PGA decreased to 100 kDa as determined by the dextran-calibrated Superose 12 column.

Purification of PA

Recombinant wild-type PA was expressed in *E. coli* BL21* (DE3) from a pET22b expression vector as described (Benson, *et al., Biochemistry 37*:3941-3948 (1998)). PA was purified from periplasmic proteins via Q Sepharose and Superdex 200 columns. Purity and molecular size of PA were verified by SDS PAGE analysis.

Synthesis of PGA-protein conjugates

Either 1.0 mg (for preparation of DAAV-1) or 0.5 mg (DAAV-2) of PA in 0.6 ml of PBS (pH 7.0) was mixed with 0.5 mg of degraded PGA. Five mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma) were added and the mixture was stirred for 4 h at room temperature. The conjugates were purified on a PD10 column, verified by SDS PAGE analysis, and stored at -20°C until use. Similarly, PGA was conjugated to bovine serum albumin (BSA) at a ratio of 1:1 (w/w) for use in antibody measurements.

Synthesis of PGA-hepatitis B core protein conjugate (PGA-HcAg). The conjugate was synthesized by the same method as described above. In brief, equal amounts (1.3 mg) of PGA and HcAg were mixed and then 5.0 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was added. The reaction was carried out at 25°C for 4h. The conjugate was verified by SDS PAGE analysis.

Mouse immunization

Groups of female BALB/c mice at 6 to 8 weeks of age (Jackson Laboratories, Bar Harbor, ME) were immunized by intraperitoneal injection on days 0, 14, and 28. DAAV-1 was tested at 10- and 20-μg doses and DAAV-2 was tested at 2-, 10-, and 20-μg doses. PA and PGA were tested at 10-μg doses. Unconjugated PGA/PA mixture included 10 μg of PGA and 10 μg of PA. Each dose was dissolved in 50 ml of PBS and adsorbed to an equal volume of $Al(OH)_3$ gel adjuvant (equivalent to 0.187 mg/dose). PBS/$Al(OH)_3$ was used as a negative control. Sera were obtained prior to immunization (preimmune) and 1 week after each vaccination.

To evaluate the PGA-HcAg conjugate, a group of 8 BALB/c mice, 6 to 8 weeks old, were immunized with 10-μg dose PGA-HcAg three times at two-week intervals. $Al(OH)_3$ was used as an adjuvant. Sera were obtained 1 week after each vaccination.

Quantification of anti-PA and anti-PGA antibodies

Serum levels of anti-PA and anti-PGA antibodies were determined by enzyme-linked immunosorbent assays (ELISAs). In brief, Immulon 96-well Maxisorp plates (Nunc) were coated with 250 ng of PA or PGA-BSA conjugate in 0.1 M carbonate buffer (pH 9.6) at 4°C for 16 h. Serum samples were serially diluted in TBS (Tris-buffered saline; 50 mM Tris-HCl, 0.15M NaCl, pH 7.4) containing 5% (w/v) fetal calf serum with 0.05% Brij and titrated two-fold across the plates. The plates were incubated at 37°C for 1 h. After washing, bound IgG or IgM antibodies were detected with 1 mg/ml of alkaline phosphatase-labeled goat anti-mouse IgG or rat anti-mouse IgM (Southern Biotech, Birmingham, AL) at 1:2,000 dilutions. The plates were developed with *p*-nitrophenyl phosphate (Sigma) at room temperature for 30 min and their absorbances determined at 405/630 nm. Antibody isotypes were determined in a similar manner, except that alkaline phosphatase-labeled anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotech) was used as the secondary antibody, respectively. Standard curves were obtained for each plate using goat $F(ab')_2$ anti-mouse Ig as the capturing agent and known concentrations of mouse IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 as standards. Specific Ig concentrations were determined by comparison with the standard curves.

Statistical analysis

The Mann-Whitney test was employed to assess the statistical significance of differences between independent sample groups. A two-sided probability value of <0.05 was considered statistically significant.

Immunoelectron microscopy

Immunoelectron microscopy was performed to determine whether antibodies raised against PGA-PA conjugates bind to PGA capsules of *B. licheniformis* 9945a. *B. licheniformis* 9945a cells were grown in E medium broth at 37°C overnight. Drops of bacterial solution (5 µl) were placed on a piece of Parafilm and a 200-mesh Formvar carbon-coated copper grid (Electron Microscopy Sources, Fort Washington, MA) was placed on top of each drop for 1 min. The grids were blocked by placing them on a 5-µl drop of 0.5% fish skin gelatin in PBS with 0.1% Tween 20. The grids were then incubated with immune mouse sera (at dilutions of 1:5) for 20 min at room temperature and washed with PBS-Tween. Subsequently, the grids were incubated with rabbit anti-mouse antibody (at a dilution of 1:5) for 20 min and washed with PBS-Tween. Gold-labeled protein A (20-nm gold particles) was applied at a dilution of 1:10 to detect bound antibodies. After incubation for 20 min at room temperature, the grids were washed four times with deionized water. Photographs were taken on a JEOL 1200 EX electron microscope at magnifications of 10,000- to 25,000-fold. Preimmune mouse sera were tested as controls using the same procedure.

Antibody-specific and complement-mediated bacteriolysis

To evaluate the functional specificity of anti-PGA sera against bacilli, we adapted a complement-mediated bactericidal assay with slight modifications (Jennings, *et al. J. Exp. Med. 165*:1207-1211 (1987)). In brief, pooled sera from groups of immunized mice were serially diluted in PBS. Samples of 30 µl of *B. licheniformis* 9945a (~100 CFUs) in PBS were incubated with 50 µl of diluted sera and 20 µl of human complement (diluted 1:4 in PBS) at 37°C for 1 h on a platform shaker. As negative reference controls, samples of *B. licheniformis* were co-cultured with undiluted preimmune sera and complement. Bacteria were also cultured with sera or complement alone as controls. 50-µl samples of incubation mixture were plated on 5% sheep blood agar plates. After incubating the plates at 37°C overnight, the number of colonies was counted and the percentage of bacterial killing was calculated for

each antibody titration by comparison with the number of colonies on preimmune reference plates. All tests were performed in four replicates.

Inhibition of PA cytotoxicity

To evaluate the ability of anti-PA antibodies to inhibit the biological activity of PA, we performed PA-mediated cytotoxicity experiments with CHO K1 cells (Milne *et al.*, *Mol. Microbiol 15*:661-665 (1995)). Sera obtained from mice immunized with DAAV-1, DAAV-2, or PA were normalized with respect to their anti-PA IgG concentrations and serially diluted. Control sera from mice immunized with PBS or PGA were serially diluted based on the before group that had the lowest anti-PA IgG concentrations. Confluent CHO K1 cells in a 96-well plate were incubated with 0.1 nM PA, 0.1 nM LF_N-DTA, and diluted sera at 37°C for 4 h. LF_N-DTA is a fusion protein of the N-terminal PA-binding domain of LF (LF_N) to the diphtheria toxin A chain (DTA). The DTA moiety catalyzes ADP-ribosylation of elongation factor-2 within the cytosol and inhibits protein synthesis. Cytotoxicity was measured by the decrease of [^{3}H]-Leu uptake by cells. Inhibition of cytotoxicity was expressed as the percentage of incorporated radioactivity relative to control (radioactivity recovered from cells incubated without LF_N-DTA). Experiments were performed in duplicates and repeated three times.

Protection of mice against lethal toxin challenge

To verify the protective activity of PGA-PA conjugates as anthrax vaccine, we tested whether immunization with DAAVs protects mice against challenge with lethal toxin. Two weeks after the third dose of vaccination, each mouse was challenged through its tail vein with a mixture of 50 μg of PA and 20 μg of lethal factor (List Biologicals, Campbell, CA), the equivalent of approximately 4 times the LD_{50} of lethal toxin in mice (Price, *et al.*, *Infect. Immun. 69*:4509-4515 (2001)). Without exception, unprotected mice died within 24 h. Protected mice were monitored closely for at least two weeks.

Histopathological analysis

Mouse organs were fixed in Bouin's fixative for 2 days, embedded in paraffin, and 5-μm thin sections were cut and stained with hematoxylin and eosin.

B. Results

DAAV is a PGA-PA conjugate vaccine designed to optimize the immunogenicity of both poly-γ-D-glutamic acid and protective antigen. As described above, recombinant PA was prepared from *E. coli* and PGA from *B. licheniformis* ATCC 9945a. Conjugates were synthesized by coupling carboxyl groups of PGA to the amines of PA via carbodiimide-mediated condensation. Below, results are presented for two sets of conjugates with 1:2 and 1:1 (w/w) PGA-to-PA ratios, designated DAAV-1 and DAAV-2, respectively.

DAAV-1 elicited strong humoral responses against both PGA and PA in immunized mice (Figure 1). After three immunizations, DAAV-1 induced median serum anti-PGA concentrations of 62.5 μg/ml (10 μg doses) and 82.2 μg/ml (20-μg doses) (Figure 1A). Booster injections significantly enhanced the IgG response. Median anti-PGA IgM levels varied between 4.1 and 9.4 μg/ml with no booster effect (Fig. 1B). In contrast, PGA alone elicited virtually no specific IgG and barely detectable amounts of IgM (Fig. 1A-B). This result illustrates that PGA by itself is poorly immunogenic, similarly to other repetitive polymeric antigens such as bacterial polysaccharides. Co-administration of unconjugated PGA/PA mixture did not improve PGA immunogenicity, revealing that covalent linkage to an immunogenic carrier is required.

DAAV-1 induced median anti-PA IgG concentrations of 715.3 μg/ml (10-μg doses) and 527.5 μg/ml (20-μg doses) after three vaccinations (Figure 1C). Comparing the effects of the 2nd and 3rd immunizations, booster doses drastically increased specific IgG by ~10-fold over 14 days. PA alone and PGA/PA mixture reached median anti-PA levels of 426.8 and 319.9 μg/ml, respectively. Both values are statistically significantly lower than those elicited by DAAV-1. PA-specific IgM levels remained barely detectable (<1 μg/ml) in all groups (Figure 1D). Overall, the conjugation of PGA and PA in DAAV-1 synergistically enhanced the immunogenicity of both PGA and PA.

The antibody response pattern after DAAV-2 immunization resembled that of DAAV-1 but the specific IgG levels attained were significantly lower (Figure 2). At 2-, 10-, and 20-μg doses and after three injections, DAAV-2 elicited median values of 18.4, 38.1, and 36.8 μg/ml of anti-PGA IgG (Figure 2A). Notable amounts of anti-PGA IgM were detected after

two doses, rising very slightly to medians of 8.0-13.6 μg/ml after three doses (Figure 2B). DAAV-2 also induced 2.1, 48.8, and 29.6 μg/ml of anti-PA IgG after three 2-, 10-, and 20-μg doses, respectively (Figure 2C).

DAAV-1 is a more promising vaccine candidate than DAAV-2. DAAV-1 induced about twice as much anti-PGA IgG as DAAV-2 and its anti-PA IgG potency was ~10-fold superior. This difference is likely due to the lower degree of modification of PA by PGA coupling in DAAV-1. The amount of PGA coupled to PA may alter natural epitopes and affect PA immunogenicity. Specifically, PA immunogenicity in DAAV-1 (PGA:PA, 1:2) was increased compared to native PA, whereas it was decreased in DAAV-2 (PGA:PA, 1:1). These results demonstrate that the structural parameters of PGA-PA conjugates are critical determinants for the induction of PGA- and PA-specific antibodies.

Since humoral immunity can depend on IgG subclass profiles, we characterized the relative proportions of specific IgG1, IgG2a, IgG2b, and IgG3 against both PGA and PA. Anti-PGA IgG1 dominated after both DAAV-1 (98%) and DAAV-2 (98%) vaccination. Similarly, anti-PA IgG1 accounted for 99% (DAAV-1) and 98% (DAAV-2) of total specific IgG, which is consistent with the antibody response to native PA (Williamson, *et al.*, *J. Appl. Microbiol. 87*:315 (1999)).

To evaluate the dual functional potency of DAAVs against bacilli and toxins, we first examined the ability of anti-PGA antibodies to opsonize and facilitate the killing of bacilli. Due to the inaccessibility to *B. anthracis* and the hazards associated with working with this pathogen, we tested *B. licheniformis* 9455a. Encapsulated *B. licheniformis* is surrounded by a thick coat of fibrillar PGA which is chemically and immunologically identical to that of *B. anthracis* (Mesnage, *et al.*, *J. Bacteriol. 180:52* (1998); Makino, *et al.*, *J Bacteriol 171:722* 1989)). As shown by immunoelectron microscopy, anti-PGA antibodies from mice immunized with DAAV-1 or -2 bound specifically to the capsule of *B. licheniformis* and completely opsonized the bacterium. Preimmune sera or sera from mice immunized with PBS did not react with the PGA capsule at all.

After binding to the capsule, antibodies can mediate the killing of bacteria via complement-driven lysis and/or opsonophagocytosis. We performed bacteriolytic assays to

test the potency of anti-PGA antibodies to activate complement and kill bacilli (Frasch, *et al.*, *Infect Immun 5:*98 (1972); Jennings, *et al.*, *J. Exp. Med. 165:*1207 (1987)). Sera from mice immunized with either DAAV-1 or -2 exerted potent bactericidal activity (Figure 4). At 1:10 serum dilution, more than 80% of bacilli were killed under assay conditions. *B. licheniformis*, unlike *B. anthracis*, sheds large amounts of soluble PGA which consume anti-PGA antibodies and reduce lytic activity. Therefore, the bactericidal capability of anti-PGA antibodies against *B. anthracis* can be expected to be significantly higher. Bacteriolytic potency correlated with serum dilution and anti-PGA antibody concentration. Neither anti-PGA serum nor complement alone were able to kill bacilli. These functional studies demonstrate that DAAV-induced anti-PGA antibodies that destroy bacilli by antibody-specific, complement-mediated bacteriolysis. The antibacterial component of DAAVs could potentially provide a prophylactic shield against anthrax infection.

We next evaluated whether DAAV vaccination could protect against anthrax toxins. PA, the required core component of anthrax toxins, translocates both lethal and edema factor into the cytosol of host cells. Since blocking PA prevents anthrax toxicity, we tested the ability of DAAV-induced anti-PA antibodies to neutralize PA (Mourez, *et al.*, *Nat. Biotechnol. 19:*958 (2001)).We exposed CHO cells to PA and LF_N-DTA, a fusion protein of PA-binding domain of lethal factor (LF_N) and diphtheria toxin A chain (DTA). PA-mediated translocation of LF_N-DTA into the cytosol causes inhibition of protein synthesis and cell death, whereas blocking of PA prevents translocation and protects cells. As shown in Figure 5A, sera from mice immunized with DAAV-1, -2, or PA efficiently rescued cells. Preimmune sera or sera from mice immunized with PBS did not inhibit cytotoxicity. At concentrations ≥ 1 μg/ml, anti-PA IgG afforded complete protection. Furthermore, anti-PA IgG induced by DAAV-1, -2, or PA displayed very similar concentration-dependent protection curves. These findings indicate that anti-PA antibodies elicited by DAAVs or PA have the same functional potency to neutralize PA.

To directly verify the effectiveness of DAAVs in protection against anthrax, we challenged immunized mice with anthrax lethal toxin. Ten days after the third immunization, each mouse was given a mixture of 48 μg of PA and 20 μg of lethal factor through its tail vein, corresponding to ~4 times the LD_{50} of lethal toxin. Control mice immunized with PBS

or PGA died without exception within 24 h (Figure 5B). In contrast, all mice immunized with DAAV-1, -2, or PA survived the lethal challenge. Symptomatically, unprotected mice were characterized by minor initial hyperactivity, soon followed by pronounced moribund lethargy, whereas protected mice progressed through a short phase of reduced activity to full clinical recovery.

Macroscopically and histologically, unprotected mice had hypoplastic spleens and necrotic intestinal lesions. Other major organs such as kidneys, liver, lungs, heart, and brain appeared normal. Unprotected mice experienced extensive cell apoptosis in spleens and a sharp decrease in cell density, whereas DAAV-protected mice had normal spleens. Apoptosis was evidenced by chromatin condensation, nuclear fragmentation, and the presence of apoptotic bodies. In vitro, lethal toxin induces apoptosis by cleaving mitogen-activated protein kinase kinases. For the first time, our histological analysis dissected the specific pathological damage caused by lethal toxin in vivo.

C. Conclusion

In conclusion, we demonstrate that DAAVs are a new class of promising conjugate anthrax vaccines that can offer simultaneous protection against both bacilli and toxins. PGA-PA conjugation has several advantages. In analogy to the use of toxoids instead of toxins, modification of PA may reduce or abolish the pathophysiological potential of native PA and be safer for vaccine formulation. Conjugation can synergistically augment the protective humoral response to both PGA and PA. Despite the complex life cycle of *B. anthracis*, systemic anthrax disease is caused by the massive extracellular replication of bacilli and the secretion of toxins. We envision that our DAAVs can prevent or stop the disease by eliminating bacteria via anti-capsular antibodies early in the sequence of anthrax infection, well before severe bacteremia and toxemia take place. In addition, anti-PA antibodies provide a second line of defense against residual toxins. DAAVs embody the paradigm of combining both prophylactic (*i.e.*, antibacterial) and therapeutic (*i.e.*, antitoxic) components into a single vaccine. This concept may find broad application in combating infectious disease.

What is Claimed is:

1. A method of making a conjugate useful in the induction of antibody production and protection against anthrax, comprising: covalently coupling poly-γ-D-glutamic acid (PGA) with the protective antigen (PA) of anthrax toxin, wherein said PGA has an average molecular size of between 0.2 and 500 kDa.

2. The method of claim 1, wherein said covalent coupling takes place at a ratio of PGA to PA of between 1:0.05 (w/w) and 1:20 (w/w).

3. The method of claim 1, wherein said PGA has an average molecular size of between 0.5 and 350 kDa.

4. The method of claim 1, wherein said PGA has an average molecular size of between 20 and 200 kDa.

5. The method of claim 1, wherein said PGA has an average molecular size of between 80 and 120 kDa and said covalent coupling takes place at a PGA to PA ratio of between 1:0.5 (w/w) and 1:5 (w/w).

6. The method of claim 5, wherein said PGA to PA ratio is 1:2 (w/w).

7. The method of claim 1, wherein said covalent coupling is by means of a crosslinking molecule.

8. The method of claim 7, wherein said covalent coupling is by means of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

9. A conjugate made by the method of any one of claims 1-8.

10. A conjugate consisting essentially of PGA covalently coupled to PA, wherein said PGA has an average molecular size of between 0.2 and 500 kDa.

11. The conjugate of claim 10, wherein the ratio of PGA to PA in said conjugate is between 1:0.05 (w/w) and 1:20 (w/w).

12. The conjugate of claim 10, wherein said PGA has an average molecular size of between 0.5 and 350 kDa.

13. The conjugate of claim 10, wherein said PGA has an average molecular size of between 20 and 200 kDa.

14. The conjugate of claim 10, wherein said PGA has an average molecular size of between 80 and 120 kDa and said ratio of PGA to PA is between 1:0.5 and 1:5.

15. The conjugate of claim 14, wherein said ratio of PGA to PA is 1:2 (w/w).

16. The conjugate of claim 10, wherein said PGA is covalently coupled to said PA by a linker containing carbodiimide.

17. The conjugate of claim 16, wherein said PGA is covalently coupled to said PA by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

18. A conjugate consisting essentially of PGA coupled to an immunogenic protein useful in the induction of antibody against Bacillar capsule.

19. The conjugate of claim 18, wherein said protein is selected from the group consisting of: hepatitis B core antigen protein.

20. A vaccine comprising the conjugate protein of any one of claims 10-19 in a pharmaceutically acceptable carrier, wherein said conjugate protein is at a concentration effective, upon administration of one or more unit doses of said vaccine, to induce an immune response.

21. The vaccine of claim 20, further comprising an adjuvant.

22. A method of inducing an immune response in an individual, comprising administering the vaccine of claim 20 to said individual at a dosage sufficient to induce said immune response.

23. A vaccine comprising the conjugate protein of claim 9 in a pharmaceutically acceptable carrier, wherein said conjugate protein is at a concentration effective, upon administration of one or more unit doses of said vaccine to an individual to induce an immune response.

24. The vaccine of claim 21, further comprising an adjuvant.

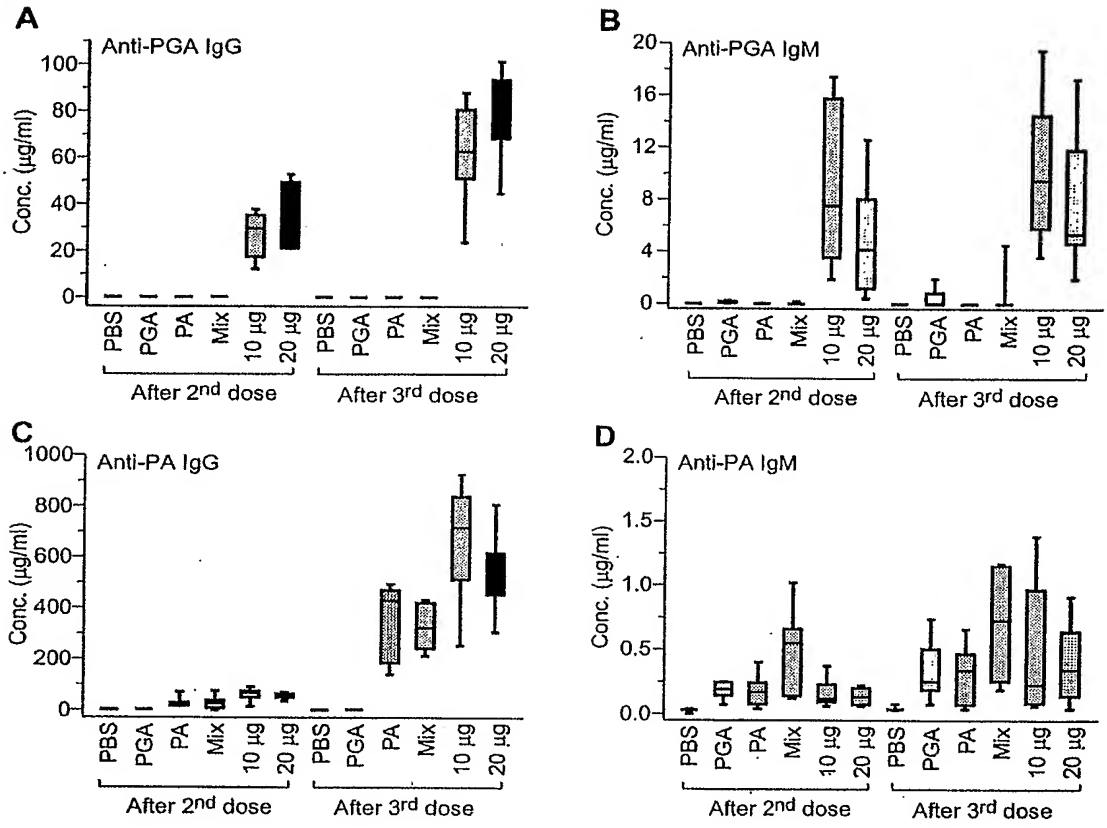
25. A method of inducing an immune response in an individual, comprising administering the vaccine of claim 21 to said individual at a dosage sufficient to induce said immune response.

26. A method of obtaining therapeutically useful antibodies comprising:
   a) administering a protein-PGA congugate to an animal capable of generating antibodies; and
   b) isolating a composition enriched in said antibodies from said animal.

27. An antibody produced by the method of claim 26.

28. A method of treating or preventing bacilli infection in a patient comprising administering an effective amount of the antibody of claim 27.

29. The method of claim 28, wherein said method is for the treatment or prevention of anthrax infection.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After 3rd dose
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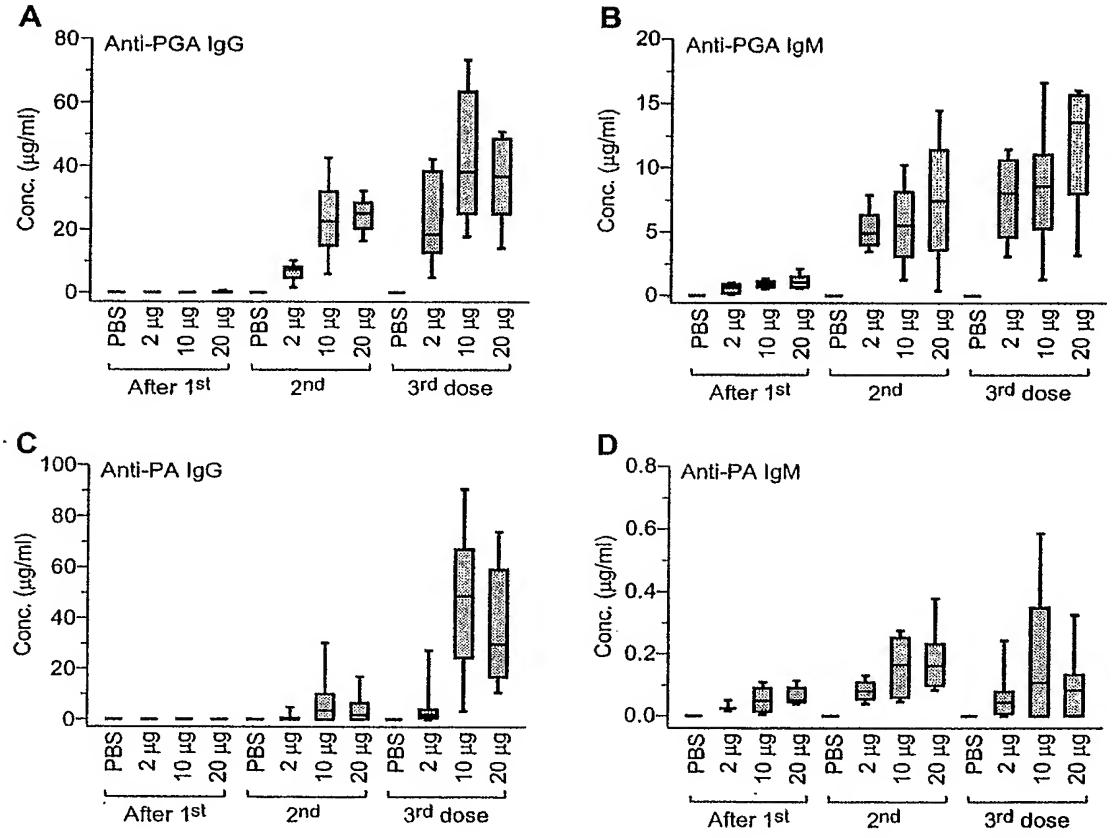
Figure 1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Conc. (µg/ml)
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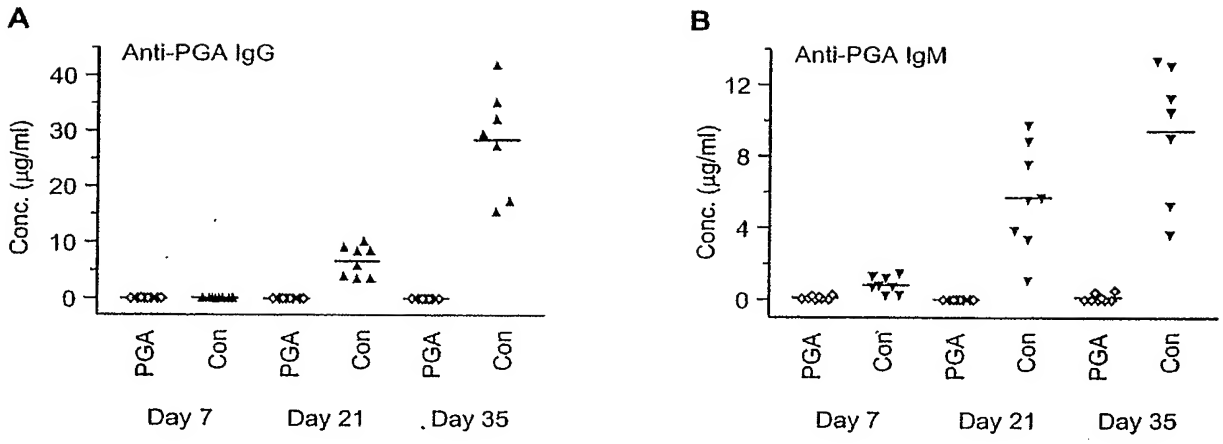
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Day 21
Day 35
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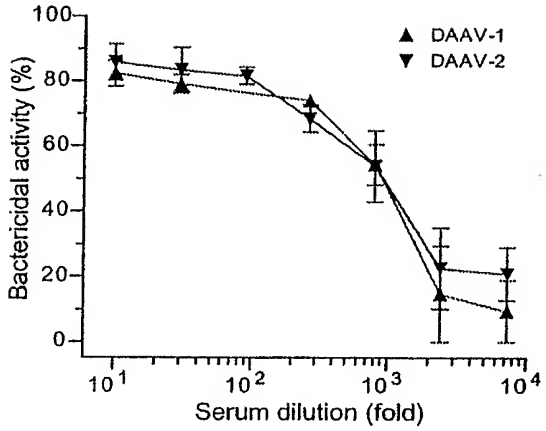
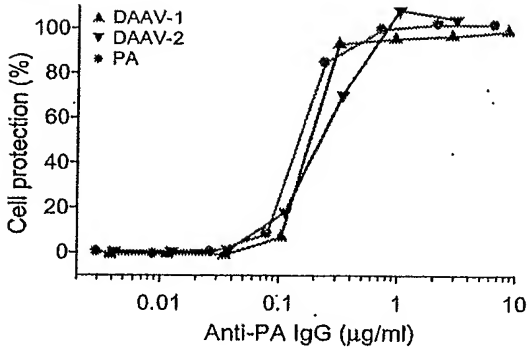


Figure 4

A



B

Immunization group	Protection against LeTx challenge (%)
DAAV-1	100 (5/5)
DAAV-2	100 (6/6)
PA	100 (6/6)
PGA	0 (0/6)
PBS	0 (0/8)

Figure 5

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Figure 6

SEQUENCE LISTING

<110> President and Fellows of Harvard College
The Brigham and Women's Hospital, Inc.
Wang, Ying
Rhie, Gi-eun
Mekalanos, John
Collier, R. John

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